Imaging skin-penetration dynamics of caffeine under various conditions by using a high-resolution non-invasive analytical method

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Summary

Background:

Evaluation of the penetration profiles of topically applied actives within the skin is essential in developing actives and cosmetic formulations. Recent advances in label-free imaging technologies have facilitated the direct detection of unlabeled chemicals in tissues. However, real-time quantitative monitoring of the penetration processes in a complex skin tissue is still a challenge. We previously developed a high-contrast, label-free skin penetration measurement system using pump-probe, phase-modulated stimulated Raman scattering (PM-SRS) microscopy and showed the skin penetration measurement of chemicals at low concentrations in an *in vitro* skin model [1,2]. However, it has not been shown that this evaluation method can also be used to *ex vivo* human skin samples and practical formulations other than aqueous solutions.

Methods:

Excised human skin samples and reconstructed human epidermis models were used for skin penetration analysis. Skin penetration profiles of caffeine were analyzed using the combined PM-SRS/confocal-reflection microscopy. Liposomes used in this study were formulated by the conventional high-pressure homogenization method.

Results:

We succeeded in direct imaging of caffeine aqueous solution and obtained penetration profiles in both *in vitro* and *ex vivo* skin measurements. The skin penetration profiles *ex vivo* showed a larger variance that in the *in vitro* measurement. In the *ex vivo* liposome measurement, it was not possible to obtain quantitative penetration profiles due to the large measurement error.

Conclusion:

We succeeded in imaging the penetration profiles of caffeine *in vitro* and *ex vivo*. Furthermore, certain challenges in improving the accuracy of the *ex vivo* evaluation system were identified.

Keywords skin penetration; label-free imaging; real-time imaging; stimulated Raman scattering microscopy

1. Introduction

The skin is the largest organ of the human body and acts as a barrier to the external environment. The outermost

upper layer of the skin, the stratum corneum (SC), consisting of corneocytes and intercellular lipids, cholesterol, free fatty acids, and ceramides, forms the essential rate-limiting barrier that prevents water loss from the body and invasion by chemicals and microbes [3,4]. Topically applied actives in cosmetics need to overcome this SC barrier to exsert beneficial effects on the skin. Therefore, understanding the penetration and distribution dynamics of applied actives is essential in cosmetics research.

Penetration analysis ideally requires time-course quantitative maps of target molecules in the skin on the same sample. There are several conventional methods for skin penetration studies, such as Franz diffusion cells and tape-stripping. The Franz diffusion cell system combines *ex vivo* skin explants or *in vitro* reconstructed human skin mounted on diffusion cells with appropriate quantification methods such as liquid scintillation spectrometry for radiation-labeled compounds [5] or high-performance liquid chromatography (HPLC) [6,7], and provides quantitative profiles of chemicals penetrating across the skin sample. Tape-stripping is a minimally invasive technique, applicable *in vivo*. Removing the SC by the repeated application of adhesive tapes and quantifying extracted target chemicals from each tape provides concentration profiles of interests across the SC [8]. Although these techniques have been validated and widely applied, they are unable to provide detailed time-course localization maps of target actives in the skin layers on the same sample.

To date, Raman spectroscopy has attracted attention as a label-free and non-invasive method in skin research. Raman spectroscopy has been applied to reveal the skin penetration profiles of topically applied substances both *in vitro* and *in vivo* [9-15] and to analyze the molecular composition of the skin [16]. However, conventional confocal Raman microscopy [9-13] often suffer from slow acquisition speeds and low signal to background ratios because of weak signals from target chemicals and strong background signals from tissues. While stimulated Raman scattering (SRS) microscopy [14,15] offers high-speed imaging, it also suffers from Raman background signals and other competing nonlinear signals. These drawbacks limit real-time imaging and signal detection at lower concentrations. In practice, a more sensitive, high-speed detection technique is required to discuss the precise penetration kinetics of actives.

Previously, we developed a novel technique, pump-probe phase-modulated (PM-) SRS microscopy, which minimizes strong background signals using pulse shaping technique and provides high-contrast imaging of small molecules in tissues [17,18]. Using this advanced technology, we reported monitoring the real-time skin penetration profiles of several molecules in an epidermis skin model [1,2]. However, it has not been shown that this evaluation method can also be used to *ex vivo* human skin samples and practical formulations other than aqueous solution.

Here, we measure the penetration of caffeine aqueous solution with *in vitro* and *ex vivo* skin samples, respectively, and discuss the differences between them. Also, we examine the penetration of caffeine in a liposome formulation with different particle sizes into human skin *ex vivo*. Through these measurements, the error factors that limit the accuracy and reproducibility of the penetration concentration measurement in human skin are clarified, and the key performance indicator of this measurement system is identified.

To date, skin penetrability of skin models with human skin and the liposome formulations have been compared with conventional methods [19,20]. Our method allows for a simple comparison of penetration behavior within skin samples.

2. Materials and Methods

2.1. Substances and formulations

Caffeine (Shizuoka Coffein, Shizuoka, Japan) was used as the target active. For an aqueous solution sample, caffeine was dissolved in pure water containing 20 % w/w propylene glycol (AGC, Tokyo, Japan) at 2% w/w.

Liposomes for this study were prepared by the conventional high-pressure homogenization method. Briefly, the lipid

composition supplied by Nippon Fine Chemical (Osaka, Japan), consisting of 80% hydrogenated soybean phospholipid and 14% phytosterol, was dispersed in glycerin (Miyoshi Oil & Fat, Tokyo, Japan) and phenoxyethanol (Clariant, Muttenz, Switzerland) by a suitable dispersion mixer. Heated water, propylene glycol and caffeine were added to the dispersion mixture and homogenized for several minutes, followed by cooling down to 35 °C under gentle stirring. The emulsion mixture was then downsized using a high-pressure homogenizer. The particle size of the liposomes was measured by a particle size analyzer (ELSZ-2000, Otsuka Electronics, Tokyo, Japan).

2.2. Sample preparation for skin penetration study

A reconstructed human skin epidermis model (EPI-606X; MatTek, Ashland, MA, USA) and a human skin sample (352 μm in thickness) of a 57-year-old Caucasian woman purchased from Biopredic International (Rennes, France) via KAC (Kyoto, Japan) were used for the skin penetration study. Biopredic International and KAC comply with the ethical standards for the acquisition, handling, selling and shipping of human products for research.

The skin model (20 mm in diameter) or excised skin (18 mm in diameter) was mounted on a glass slide with a single cavity. The space between the lower basal cell layer of the tissue and the surface of the glass slide cavity was filled with the receiver solution, 20 μL phosphate-buffered saline. A 1-mm-thick steel washer was placed on the skin model, and 50 μL of each formulation sample of caffeine was dropped directly on the surface within the inner hole of the washer. A coverslip was placed on top of the washer to enclose the solution. The interfaces between the washer and the skin model and between the washer and the coverslip were sealed with silicone grease. During the skin penetration measurements, the sample temperature was kept at 32 °C. Each sample was placed on the stage of the PM-SRS microscope to obtain data for each time.

2.3. PM-SRS imaging

Figure1 shows a schematic of the combined PM-SRS/confocal reflection microscope. The microscopy setup is designed to allow switching between the PM-SRS mode, for tracing Raman signals of target molecules, and the confocal-reflection mode, for acquiring morphological information of the tissue sample. The principles of PM-SRS and the detailed setup of the PM-SRS microscope here have been previously described [17,18]. Succinctly, a femtosecond pulse generated by a Ti-sapphire laser (Vitara, Coherent: 80 MHz, 15 fs, centered at 790 nm, exposure power 50 mW) is split into pump and probe pulses, the latter of which is further divided into two probe pulses. Excitation of the sample by a pump pulse initiates the coherent vibration of the Raman modes, and a specific target mode is detected by adjusting the difference in frequencies between the two probes. The pump and probe pulses are optimized to provide a superior signal-to-background ratio, which is achieved by selectively probing a long-lived vibrational signal from caffeine, while efficiently rejecting background signals from tissue Raman scattering and other nonlinear optical responses. To optimize the signal-to-background ratio, both probe pulses are delayed with respect to the pump pulse by 1.7 ps, and then stretched and shaped to form a pair of time-asymmetric picosecond pulses, with distinct center frequencies. In the SRS detection mode, one of the probe pulses is modulated by an electro-optic modulator (EO-PM-NR-C1, Thorlabs), whereas the non-modulated probe pulse is defined as the local oscillator (LO). All laser pulses are combined and focused onto the sample with an objective lens (LCPLN50XIR, NA 0.65, Olympus). The heterodyne SRS output signal on the transmission LO probe pulse, whose intensity is proportional to the concentration of the target molecule, is then detected by a Si photodetector (PDA36A, Thorlabs) and also demodulated with a lock-in amplifier (LI5640, NF Corporation). For the confocal-reflection microscopy mode, back-scattered light is collected by the objective lens, and the intensity is measured by another identical Si photodetector. Two-dimensional depth-resolved images are recorded via XZ raster scanning, by driving the sample stage. The data acquisition period per each XZ SRS image is 100 s.

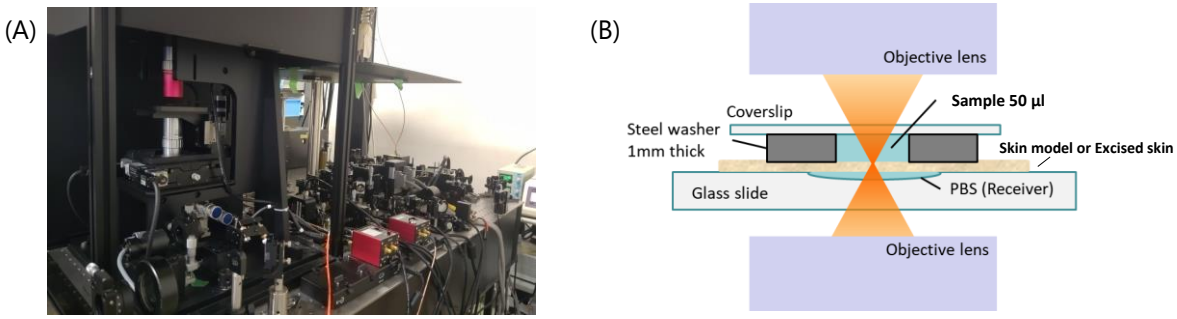

Figure 1 (A) PM-SRS microscope system. (B) Sample configuration for microscopy observation.

3. Results

3.1. Skin Penetration profiles of caffeine *in vitro*

The penetration profiles of the skin model applied with a 2% aqueous caffeine solution were measured by the PM-SRS microscopy with confocal-mode over time (n=2). The confocal reflection XZ images and the PM-SRS images at 560 cm^{-1} of the skin model acquired 30 min and 360 min after applying the solution are shown in Figures 2A to 2D as representative data. Strong confocal reflection signals were observed at the upper and lower depth position of the skin model, owing to the high refractive-index mismatch of the skin model and solution. The upper skin boundary of the PM-SRS image was defined as the position of the uppermost edge of the confocal reflection signals obtained at the same point as the relative zero-depth. The relative zero-depth was compared to the confocal Raman signal at 1004 cm^{-1}, which is the ring-breathing mode in aromatic amino acids of skin model component [22], or the optical scattering loss profile, and the relative surface position was adjusted for any shifts. As we previously reported [1], PM-SRS measurement with a 1.7ps probe delay suppressed background signals from skin model components and detected the characteristic signal from caffeine at 560 cm^{-1} (vibration from the O=C-N deformation [17]) (data not shown). The PM-SRS images show high-contrast two-dimensional distribution maps of caffeine. Although the PM-SRS signal of caffeine at 560 cm^{-1} was detected at the surface of the skin model at 30 min, the signal was observed deeper in the skin model at 360 min.

To quantitatively analyze the skin penetration profile, the SRS signal intensity accumulated twice for each time point was normalized by the signal level of 2% caffeine solution and subtracted with the base profile (Figure 2E), where the baseline is calculated by convolution of a step function (1 for Z<0 and 0 for Z>0) with the instrument response function (Lorentz function). The relative intensity was then integrated every 10 µm starting at a relative surface of 5 µm (Figure 2F). The results showed that the 5-15µm distance from the relative surface was mostly saturated in 30 minutes, with increasing concentrations in the layer lower down over time.

3.2. Skin Penetration profile of caffeine *ex vivo*

As done in the skin model measurement, 2% caffeine solution was applied to excised skin samples and measured using the PM-SRS/confocal-reflection microscopy over time (n=2). Figures 3A to 3D show the confocal reflection XZ images and the caffeine PM-SRS XZ images acquired at 560 cm^{-1}, at 60 min and 390 min after applying the solution, respectively. The quantitative profiles of caffeine were calculated on the basis of SRS signals accumulated four to eight times for each time point. The depth profiles were derived every 10 µm starting at a relative surface of 5 µm, as in the skin model experiments (Figure 3E, n=2). These data show that the PM-SRS signal of caffeine was detected in excised skin samples and the real-time penetration of caffeine was successfully imaged directly. The amount of caffeine penetrated in the skin sample after 24 hours was less than half that of in the skin model. In addition, variation in

measurements at each time point were greater *ex vivo* than *in vitro*.

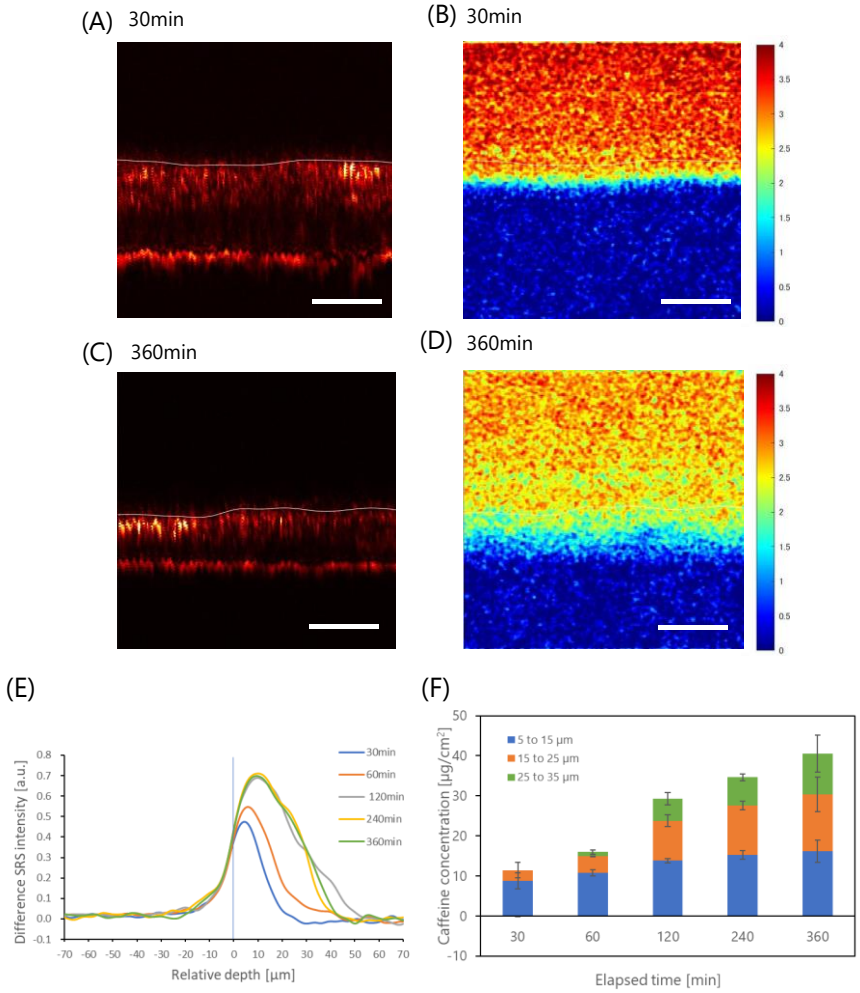

Figure 2 (A)-(D) Confocal reflection images of the XZ section of a skin model (A) 30 min and (C) 360 min after treated with 2% caffeine solution and the PM-SRS images at 560 cm^{-1} of the XZ section of a skin model with the zero-depth boundary detected with the confocal reflection contrast (dot-line) (B) 30 min after or (D) 360 min after treated with 2% caffeine solution. (E) Caffeine depth profiles in the skin model (30-360 min). (F) Concentration changes of caffeine over time. Error bars represent twice the standard error. Scale bar = 50 μm

3.3. Liposome formulation

Liposomes of three different diameters (L1, L2, and L3) were prepared by homogenization and high-pressure processing. The size distributions of each sample were measured by a particle size analyzer. The average sizes were 325.0 nm (L1), 178.9 nm (L2) and 107.5 nm (L3) respectively.

3.4. Skin penetration profiles of caffeine from liposomes

The skin penetration profiles of caffeine from L1, L2, and L3 were obtained *ex vivo* over time (n=2). Figure 4 shows the penetration profiles of L2. Even though the same formulation sample and skin samples from the same donor were used, the acquired data showed the different depth profiles. Similar variations were observed in the application of other liposome samples, and it was not possible to discuss the penetration behavior between the samples.

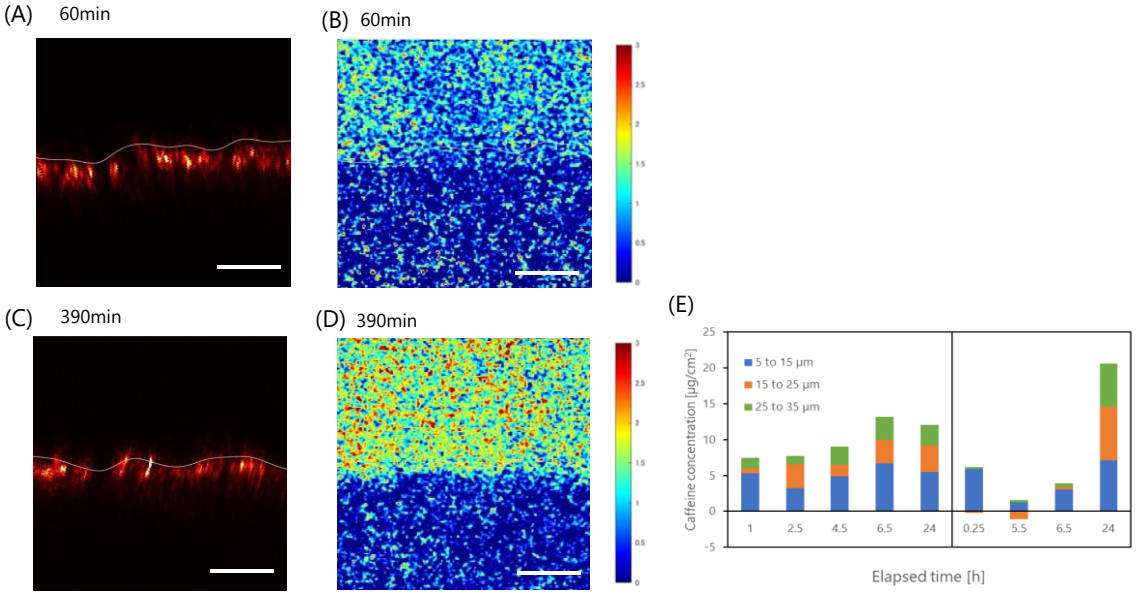

Figure 3 Confocal reflection images of the XZ section of skin excised skin (A) 60 min and (C) 390 min after treated with 2% caffeine solution. PM-SRS images at 560 cm^{-1} of the XZ section with confocal reflection boundary (dot-line) (B) 60 min after or (D) 390 min after treated with 2% caffeine solution. (E) Concentration changes of caffeine over time (n=2). Scale bar = 50 μm

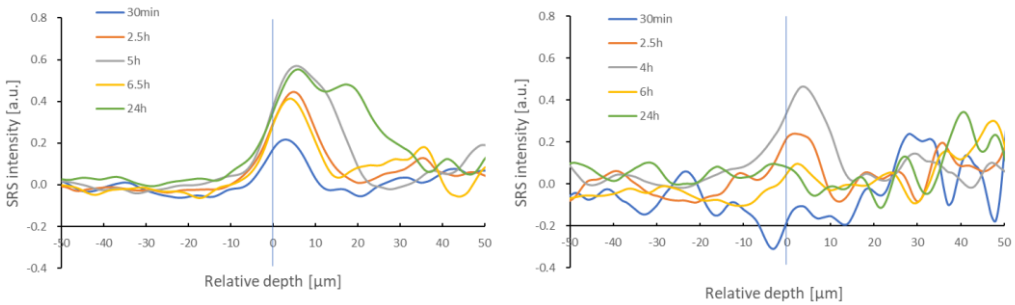

Figure 4 Caffeine depth profiles from L2 in excised skin over time (n=2).

4. Discussion

We have previously demonstrated that PM-SRS can be used as a simple, real-time imaging skin penetration evaluation system *in vitro*, providing direct quantitative depth profiles of target molecules even at lower concentrations by suppressing tissue-oriented background signals [1,2].

In this study, we first evaluated the skin penetration of aqueous and liposomal formulations of caffeine using *ex vivo* human skin. While aqueous caffeine measurements provided clear depth-resolved images and depth profiles in both *in vitro* and *ex vivo* cases, *ex vivo* liposome measurements did not provide reproducible results to discuss the penetration characteristics. The main factor limiting the accuracy of the *ex vivo* PM-SRS measurements was the low signal-to-noise ratio (S/N) of the caffeine Raman signal due to scattering losses. Thus, the S/N is the key performance indicator of this measurement system. We reported that the lower limit of detection for caffeine in this measurement

system is 0.1% [1]. In this case study of a 2% caffeine solution, to achieve this lower detection limit, at least S/N > 20 is required in the applied solution area (Z<0). In addition, the actual *ex vivo* measurements may have a lower S/N due to variability factors such as the scattering coefficient of the skin tissue and applied sample formulation, skin thickness, stratum corneum barrier condition, and their position dependence. Therefore, it is necessary to design an evaluation plan (sample concentration, exposure time and signal accumulation) to meet a sufficient S/N, which is several times more than 20, by taking these loss factors into accounts.

Notably, there may be another error factor besides the scattering loss. In the measurement of L2 liposome sample, the results of two penetration tests were different despite the relatively high S/N. Besides, at several measurement time points, the differential Raman signal (the amount of penetration) decreased with time, sometimes showing negative values. These results suggest that there was an error in the detected depth position of the sample surface boundary. One possible reason for the boundary detection error is the drift of the sample during measurement. The human skin sample used had a relatively larger depth of surface wrinkles and a greater overall thickness, which may have resulted in a larger drift during measurement compared to the skin model. This problem can be solved by holding the skin sample with appropriate tension.

5. Conclusion

In this study, we demonstrated a label-free microscopy-based method to visualize the distribution maps of topically applied caffeine in a skin model and excised human skin. PM-SRS microscopy provided high-contrast images of target molecules within skin samples. By using the combined confocal reflection and PM-SRS microscopy, we successfully imaged real-time skin penetration of caffeine *in vitro* and *ex vivo*. Through these measurements, the key performance indicator and some important error factors in the *ex vivo* penetration measurement were identified.

Compared to the *in vitro* measurement, the *ex vivo* measurement was found to have more error factors due to the complexity of the real human skin structure. A more reproducible evaluation test with quantitative skin penetration profile measurements can be designed by using S/N as an indicator. Another important technical issue is to improve the position accuracy of the surface boundary detection. By solving these issues, this microscopy-based direct measurement of chemical concentration in skin will be a practical evaluation method to understand the skin penetration of small molecule active ingredients.

Conflict of Interest Statement

The authors declare no conflicts of interest associated with this manuscript.

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